

Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2399-2403, March 1992
Medical Sciences

A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure

(chemoprotection/enzyme induction/isothiocyanates/sulforaphane/quinone reductase)

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ABSTRACT Consumption of vegetables, especially crucifers, reduces the risk of developing cancer. Although the mechanisms of this protection are unclear, feeding of vegetables induces enzymes of xenobiotic metabolism and thereby accelerates the metabolic disposal of xenobiotics. Induction of phase II detoxication enzymes, such as quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] and glutathione S-transferases (EC 2.5.1.18) in rodent tissues affords protection against carcinogens and other toxic electrophiles. To determine whether enzyme induction is responsible for the protective properties of vegetables in humans requires isolation of enzyme inducers from these sources. By monitoring quinone reductase induction in cultured murine hepatoma cells as the biological assay, we have isolated and identified (–)-1-isothiocyanato-(4*R*)-(methylsulfinyl)butane [$\text{CH}_3\text{—SO—}(\text{CH}_2)_4\text{—NCS}$, sulforaphane] as a major and very potent phase II enzyme inducer in SAGA broccoli (*Brassica oleracea italica*). Sulforaphane is a monofunctional inducer, like other anticarcinogenic isothiocyanates, and induces phase II enzymes selectively without the induction of aryl hydrocarbon receptor-dependent cytochromes P-450 (phase I enzymes). To elucidate the structural features responsible for the high inducer potency of sulforaphane, we synthesized racemic sulforaphane and analogues differing in the oxidation state of sulfur and the number of methylene groups: $\text{CH}_3\text{—SO}_n\text{—}(\text{CH}_2)_m\text{—NCS}$, where $m = 0, 1, \text{ or } 2$ and $n = 3, 4, \text{ or } 5$, and measured their inducer potencies in murine hepatoma cells. Sulforaphane is the most potent inducer, and the presence of oxygen on sulfur enhances potency. Sulforaphane and its sulfide and sulfone analogues induced both quinone reductase and glutathione transferase activities in several mouse tissues. The induction of detoxication enzymes by sulforaphane may be a significant component of the anticarcinogenic action of broccoli.

Individuals who consume large amounts of green and yellow vegetables have a lower risk of developing cancer (1–3). Feeding of such vegetables to rodents also protects against chemical carcinogenesis (4, 5), and it results in the induction in many tissues of phase II enzymes—e.g., quinone reductase [QR: NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] and glutathione S-transferases (EC 2.5.1.18) (11–17). Although much evidence suggests that induction of these enzymes is a major mechanism responsible for this protection (18–20), the precise rule of enzyme induction in protection of humans requires clarification. The preceding report (21) shows that measurement of QR activity in Hepa 1c1c7 murine hepatoma cells provides a rapid, reliable, and convenient index of phase II enzyme inducer activity in vegetables. Using this assay (21–24), we found that cruciferous vegetables (broccoli, cauliflower, mustard, cress, brussels sprouts) were a rich source of inducer activity. We chose to investi-

gate broccoli (*Brassica oleracea italica*) specifically because it is consumed in substantial quantities by Western societies and has been shown to contain abundant phase II enzyme inducer activity (21). In this paper we describe the isolation and identification of a potent major phase II enzyme inducer from broccoli.

MATERIALS AND METHODS

Source of Vegetables and Preparation of Extracts. SAGA broccoli was grown by Andrew Ayer (Maine Packers, Caribou, ME). SAGA is synonymous with Mariner broccoli (Petoseed, Arroyo Grande, CA) and was adapted for growing in Maine by Smith, Ayer, Goughan, and Arrow. The broccoli was harvested when ripe, frozen immediately, shipped to our laboratory in dry ice, and stored at -20°C until processed.

For preliminary survey of inducer activity in broccoli samples, florets were homogenized with 2 vol of water at 4°C , and the resultant soups were lyophilized to give powders, which were stored at -20°C . Portions (400 mg) of these powders were extracted for 6 hr with 14 ml of acetonitrile in glass-stoppered vessels on a wrist-action shaker at 4°C . The extracts were filtered through a sintered glass funnel and evaporated to dryness in a rotating evaporator ($<40^\circ\text{C}$). The residues were dissolved or suspended in 100 μl of dimethyl formamide and assayed for inducer activity.

Assay of Inducer Activity. Inducer potency for QR was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates (21, 24). The cells (10,000 per well) were grown for 24 hr and then exposed to inducer for 48 hr. Usually 20 μl of the solutions to be assayed (in acetonitrile or dimethyl formamide) was added to 10.0 ml of medium and 2-fold serial dilutions were used for the microtiter plates. The final organic solvent concentration was always less than 0.2% by volume. One unit of inducer activity is defined as the amount that when added to a single microtiter well (containing 150 μl of medium) doubles the QR specific activity. The inducer potency of compounds of known structure has been determined in the above system also, and it is expressed as

Abbreviations: QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2]; CD value, the concentration of a compound required to double the quinone reductase specific activity in Hepa 1c1c7 murine hepatoma cells.

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‡Enzymes of xenobiotic metabolism belong to two families (6): (i) phase I enzymes (e.g., cytochromes P-450), which functionalize compounds, usually by oxidation or reduction; although their primary role is to detoxify xenobiotics, several cytochromes P-450 can activate procarcinogens to highly reactive ultimate carcinogens (7); and (ii) phase II enzymes, which conjugate functionalized products with endogenous ligands (e.g., glutathione, glucuronic acid, sulfate) and play primarily a detoxication role (8). QR is considered a phase II enzyme because it serves protective functions (9), is induced coordinately with other phase II enzymes, and is regulated by enhancer elements similar to those that regulate glutathione transferases (10).

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the concentration required to double (CD value) the QR activity.

The inductions of QR and glutathione transferase activities in mouse organs were studied according to a standard protocol (25).

Synthesis of Compounds. (*R,S*)-Sulforaphane (CAS 4478-93-7) was prepared according to Schmid and Karrer (26) except that gaseous thiomethanol was replaced by sodium thiomethoxide. The sulfide analogues, $\text{CH}_3\text{—S—(CH}_2\text{)}_n\text{—NCS}$, where n is 4 [erucin (CAS 4430-36-8)] or 5 [berteroin (CAS 4430-42-6)] were prepared as described (27), and the three-carbon analogue [iberverin (CAS 505-79-3)] was prepared from phthalimidopropyl bromide (26). IR spectra of all three sulfide analogues showed strong absorptions near 2150 cm^{-1} , characteristic of isothiocyanates. $^1\text{H NMR}$ spectra of these compounds show sharp singlets at $\delta\ 2.10\text{ ppm}$ ($\text{CH}_3\text{—S—}$ group). The sulfoxide analogues where n is 3 [iberin (CAS 505-44-2)] or 5 [alyssin (CAS 646-23-1)] were prepared by the same method as sulforaphane. IR spectra of these compounds showed strong absorptions near 2100 cm^{-1} , assigned to the —NCS group. $^1\text{H NMR}$ spectra also showed sharp singlets around $\delta\ 2.5\text{ ppm}$, consistent with the presence of the $\text{CH}_3\text{—SO—}$ group. The sulfone analogues, $\text{CH}_3\text{—SO}_2\text{—(CH}_2\text{)}_n\text{—NCS}$, where n is 3 [cheirolin (CAS 505-34-0)], 4 [erysolin (CAS 504-84-7)], or 5 (unreported) were prepared by known methods (28). $^1\text{H NMR}$ ($\delta = 2.9\text{ ppm}$, for $\text{CH}_3\text{—SO}_2\text{—}$) and IR spectra of these compounds were consistent with the structures. Every analogue except 1-isothiocyanato-5-methylsulfonylpentane [$\text{CH}_3\text{—SO}_2\text{—(CH}_2\text{)}_5\text{—NCS}$] has been isolated from plants (29).

RESULTS

Isolation of Inducer Activity. We selected SAGA broccoli for study because acetonitrile extracts of lyophilized homogenates of this variety were especially rich in inducer

activity (62,500 units per g) in comparison with other vegetables (21). Fractionation of acetonitrile extracts of SAGA broccoli by preparative reverse-phase HPLC (Fig. 1) with a water/methanol solvent gradient resulted in recovery of 70–90% of the applied inducer activity in the chromatographic fractions. Surprisingly, the majority (about 65–80% in several chromatographies) of the recovered activity was associated with a single and relatively sharp peak [fractions 18–23; eluted at 64–71% (vol/vol) methanol]. This HPLC procedure was therefore adopted as the first step of the larger-scale isolation of inducer activity.

Lyophilized SAGA broccoli was extracted three times with acetonitrile (35 ml/g) for 6 hr each at 4°C . The pooled extracts were filtered and evaporated to dryness under reduced pressure on a rotating evaporator ($<40^\circ\text{C}$). About 1 g of residue from 640 g of fresh broccoli (64 g of lyophilized powder) contained 3.6×10^5 units of inducer activity. The residue was mixed thoroughly with 120 ml of methanol/water (25/75, vol/vol) and the insoluble fraction was discarded. Although not all of the residue obtained from the extraction was soluble in aqueous methanol, the solvent partition procedure resulted in substantial purification without significant loss of inducer activity. Portions of the extract were dried in a vacuum centrifuge and dissolved in small volumes of dimethyl formamide (0.75–1.0 ml per 50 mg of residue), and 50-mg portions were subjected to HPLC (nine runs) as described in the legend of Fig. 1. Fractions 18–23 from all runs were pooled, evaporated to dryness, applied in acetonitrile to five preparative silica TLC plates ($100 \times 200 \times 0.25\text{ mm}$), and developed with acetonitrile, which was run to the top of each plate three times. Four major fluorescence-quenching components were resolved, and nearly all (99%) of the inducer activity migrated at $R_f\ 0.4$. The active bands were eluted with acetonitrile, pooled, and fractionated by two runs on a second preparative reverse-phase HPLC in a water/

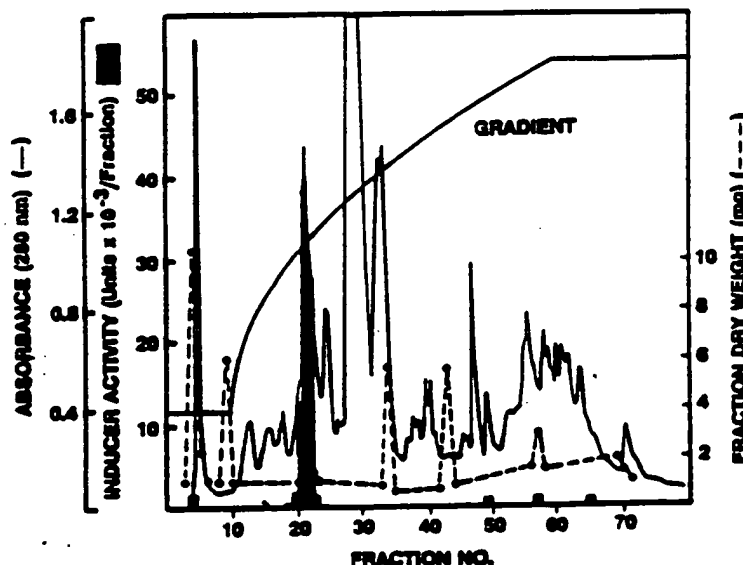


FIG. 1. Reverse-phase HPLC of acetonitrile extract of SAGA broccoli showing the distribution of absorbance at 280 nm, total inducer activity (units per fraction), and dry weight of each fraction. Lyophilized SAGA broccoli flower powder (16 g) was extracted three times (for 6 hr each) with 560-ml portions of acetonitrile on a shaker at 4°C . The extracts were filtered and evaporated to dryness on a rotating evaporator ($<40^\circ\text{C}$). The residue (202 mg) was suspended in 3.0 ml of methanol and filtered successively through 0.45- and 0.22- μm porosity filters. The insoluble material was discarded. The filtrate was assayed for total inducer activity, and a 0.75-ml (50.5-mg) aliquot of the methanol extract was subjected to HPLC on a reverse-phase preparative column (Whatman; Partisil 10 ODS-2; $50 \times 1.0\text{ cm}$) equilibrated with methanol/water (30/70, vol/vol), eluted at a rate of 3.0 ml/min, and 6.0-ml fractions were collected. Elution solvent: 30 ml of initial solvent, followed by 330 ml of a convex gradient (Waters Gradient program 5) to 100% methanol, and then by 90 ml of 100% methanol. The fractions were evaporated on a vacuum centrifuge (Savant Speed-Vac Concentrator), and the residues were weighed, redissolved in 0.1 ml of dimethyl formamide, and assayed for inducer activity. The activity applied (0.75 ml = 104,000 units) was recovered principally in fractions 18–23 (84,600 units, 81%), and minor amounts of activity were found in fractions 4, 49, 57, and 65. The total recovery of inducer activity in all fractions was 90% of that applied to the column.

acetonitrile gradient (Fig. 2). Ultraviolet absorption and inducer activity were eluted in a sharp coincident peak (at 66% acetonitrile) that contained all of the activity applied to the column. Evaporation (<40°C) of the active fractions gave 8.9 mg of a slightly yellow liquid, which contained 558,000 inducer units (overall yield 15%) and migrated as a single band on TLC.

Identification of Inducer. The identity of the inducer was established by spectroscopic methods and confirmed by chemical synthesis. It is (-)-1-isothiocyanato-(4*R*)-(methylsulfinyl)butane, known as sulforaphane or sulphoraphane (CAS 4478-93-7):



Sulforaphane has been synthesized (26) and isolated from leaves of hoary cress (30) and from other plants (31), and the absolute configuration has been assigned (32). The closely related olefin sulforaphene [4-isothiocyanato-(1*R*)-(methylsulfinyl)-1-(*E*)-butene (CAS 2404-46-8)] has been isolated from radish seeds and other plants (33, 34) and has also been synthesized (35, 36).

The following evidence establishes that (*R*)-sulforaphane is the inducer isolated from broccoli. UV spectrum (H₂O): λ_{max} 238 nm, ϵ_{238} 910 M⁻¹cm⁻¹; addition of NaOH (0.1 M) blue-shifted (λ_{max} 226 nm) and intensified (ϵ_{226} 15,300 M⁻¹cm⁻¹) this absorption band, consistent with the behavior of isothiocyanates (37). IR (Fourier transform, neat): strong absorptions at 2179 and 2108 cm⁻¹ and also at 1350 cm⁻¹, characteristic of isothiocyanates (27). ¹H NMR (400 MHz,

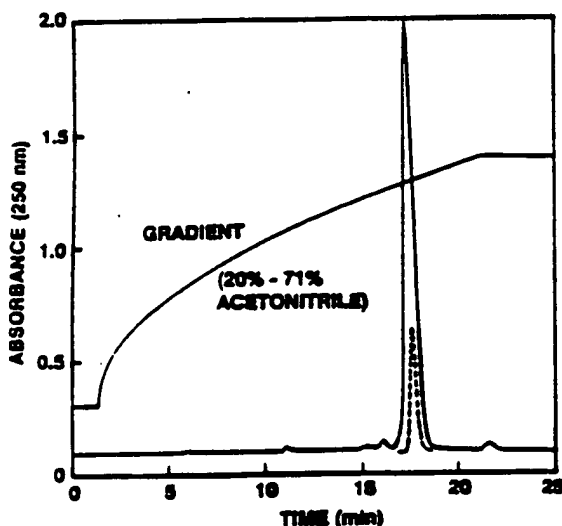
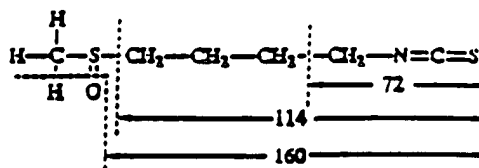


FIG. 2. Second reverse-phase preparative HPLC of enzyme inducer activity from SAGA broccoli. The active inducer bands obtained from two or three preparative silica TLC plates (see text) were combined, eluted with acetonitrile, filtered twice through 0.22- μ m porosity filters, and evaporated to dryness on a vacuum centrifuge. The residue was dissolved in 0.5 ml of acetonitrile and applied to a reverse-phase preparative HPLC column (Whatman: Partisil DS-2; 50 \times 1.0 cm), which was developed with a convex gradient (Waters Gradient program 5) of acetonitrile/water from 20:80% to 71:29% (vol/vol) at a flow rate of 3.0 ml/min during a 20-min period. The eluate from 17.0 to 19.0 min was collected as a pool and assayed for inducer activity; 99% of the inducer activity was recovered in this pool. The elution position of (*R,S*)-sulforaphane is shown (—).

¹HCl₃): δ 3.60 (t, 2H, $J = 6.1$ Hz, —CH₂—NCS), 2.80–2.66 (m, 2H, —CH₂—SO—), 2.60 (s, 3H, CH₃—SO—), and 1.99–1.86 ppm (m, 4H, —CH₂CH₂—). ¹³C NMR (400 MHz, C¹HCl₃): δ 53.5, 44.6, 38.7, 29.0, and 20.1 ppm. Mass spectrometry (fast atom bombardment; thioglycerol matrix) gave prominent peaks at 178 (M + H)⁺ and 355 (M₂ + H)⁺. Electron impact mass spectrometry gave a small molecular ion (M⁺) at 177, and chemical ionization mass spectrometry gave a small molecular ion (M + H)⁺ at 178 and prominent fragment ions with masses of 160, 114, and 72, consistent with the following fragmentation:



Precise masses of molecular and fragment ions obtained by electron impact mass spectrometry were 177.0286 (calculated for C₆H₁₁NOS₂, 177.0283), 160.0257 (calculated for C₄H₁₀NS₂, 160.0255), and 71.9909 (calculated for C₂H₂NS₁, 71.9908). In addition, for the mass 160 fragment, the peaks at 161 (M + 1) and 162 (M + 2) were 8.43% (calculated, 8.44%) and 9.45% (calculated, 10.2%), respectively, of the parent ion. Similarly, for the mass 72 fragment, the peaks at 73 (M + 1) and 74 (M + 2) were 3.42% (calculated, 3.32%) and 5.23% (calculated, 4.44%), respectively, of the parent ion. Hence the isotope compositions corrected for the natural isotopes abundance (of ¹³C, ¹⁵N, ³³S, and ³⁴S) were consistent with the relative intensities of the M + 1 and M + 2 ions of both fragments. The optical rotation of the isolated material was [α]_D²⁰ -63.6° (c = 0.5, CH₂Cl₂), thus establishing that the product is largely, if not exclusively, the (-)-(*R*) enantiomer (literature [α]_D²⁰ -79°, -73.2°, -66°; refs. 26, 30, and 38, respectively). The spectroscopic properties of synthetic (*R,S*)-sulforaphane were identical to those of the isolated product.

Relation of Structure to Inducer Activity Among Sulforaphane Analogues. To define the structural features of sulforaphane (chirality, state of oxidation of sulfur of the thiomethyl group, number of methylene bridging groups) important for inducer activity, we synthesized (*R,S*)-sulforaphane and the following analogues and measured their inducer potencies: CH₃—S—(CH₂)_n—N=C=S (n = 3, 4, or 5); CH₃—SO—(CH₂)_n—N=C=S (n = 3 or 5); and CH₃—SO₂—(CH₂)_n—N=C=S (n = 3, 4, or 5).

Induction of QR in Murine Hepatoma Cells. The chirality of the sulfoxide does not affect inducer potency, since isolated (*R*)-sulforaphane and synthetic (*R,S*)-sulforaphane gave closely similar CD values of 0.4–0.8 μ M. Sulforaphane is therefore the most potent monofunctional (see below) inducer that has been identified (19, 20). Both (*R*)- and (*R,S*)-sulforaphane were relatively noncytotoxic; the concentrations required to depress cell growth to one-half were 18 μ M.

Sulforaphane and the corresponding sulfone (erysolin) were equipotent as inducers of QR, whereas the corresponding sulfide (erucin) was about one-third as active (Table 1). Oxidation of the side-chain sulfide to sulfoxide or sulfone enhanced inducer potency, and compounds with 4 or 5 methylene groups in the bridge linking CH₃— and —N=C=S were more potent than those with 3 methylene groups (Table 1).

Mutants of Hepa 1c1c7 cells defective in the Ah (aryl hydrocarbon) receptor or expression of cytochrome P-450IA1 can distinguish monofunctional inducers (which induce phase II enzymes selectively) from bifunctional inducers

Table 1. Potency of induction of QR in Hepa 1c1c7 cells by sulforaphane and analogues

Compound	CD value, μM		
	<i>n</i> = 3	<i>n</i> = 4	<i>n</i> = 5
$\text{CH}_3\text{—S—(CH}_2\text{)}_4\text{—N=C=S}$	3.5 (Iberverin)	2.3 (Erucin)	1.7 (Berteroin)
$\text{CH}_3\text{—S—(CH}_2\text{)}_3\text{—N=C=S}$	2.4 (Iberin)	0.4–0.8 (Sulforaphane)	0.95 (Alyssin)
$\text{CH}_3\text{—S(=O)—(CH}_2\text{)}_4\text{—N=C=S}$	1.3 (Cheirulin)	0.82 (Erysolin)	0.98

Trivial names are given in parentheses. See Kjær (29).

ducers (which elevate both phase I and II enzymes) (39, 40). When sulforaphane was tested with the BP^c1 mutant (41) (defective in transport of the liganded Ah receptor to the nucleus), and the c1 mutant (42) (which synthesizes inactive cytochrome P-450IA1), induction of QR was normal (data not shown). Sulforaphane is, therefore, like benzyl isothiocyanate, a monofunctional inducer (40) and is unlikely to elevate activities of cytochromes P-450 that could activate carcinogens.

Induction of QR and Glutathione Transferase Activities in Mice. When synthetic (*R,S*)-sulforaphane, crysolin, and erucin were administered to female CD-1 mice by gavage (25), induction of QR and glutathione transferase activities was observed in the cytosols of several organs (Table 2). Sulforaphane and erucin (in daily doses of 15 μmol for 5 days) raised both enzyme activities 1.6- to 3.1-fold in liver, forestomach, glandular stomach, and mucosa of proximal small intestine, and to a lesser degree in lung. The sulfone (erysolin) was more toxic, but even 5- μmol daily doses for 5 days elevated the specific activities of these enzymes in some tissues examined. We therefore conclude that sulforaphane and its analogues not only induce QR in Hepa 1c1c7 murine hepatoma cells but also induce both QR and glutathione transferase activities in a number of murine organs.

DISCUSSION

Two considerations prompt the belief that sulforaphane is a major and probably the principal inducer of phase II enzymes present in extracts of SAGA broccoli. First, high yields of

inducer activity were obtained at each step of the isolation, and even in the first HPLC (Fig. 1) more than 60% of the inducer activity was contained in a single chromatographic peak, the biological activity of which could not be subfractionated. Second, when a totally independent method of isolation of inducer activity by high-vacuum sublimation of lyophilized broccoli (5 μm Hg pressure, 60–165°C, condenser at –15°C) was used, nearly all the isolated inducer activity was found in the methanol-soluble portion of the sublimate. Moreover, on HPLC (Fig. 2) this sublimed material gave rise to only a single isothiocyanate-containing fraction, which on TLC comigrated with authentic sulforaphane and after further purification by TLC provided a high yield of sulforaphane characterized unequivocally by NMR.

The finding that the majority of the inducer activity of SAGA broccoli probably resides in a single chemical entity, an isothiocyanate, is of considerable interest. Isothiocyanates (mustard oils) and their glucosinolate precursors are widely distributed in higher plants and are especially prevalent among cruciferous vegetables (29). Sulforaphane has been identified in species of *Brassica*, *Eruca*, and *Iberis* (29, 31).

Isothiocyanates have been shown to block chemical carcinogenesis. In rats, 1-naphthyl isothiocyanate suppressed hepatoma formation by 3-methylcholanthrene, 2-acetylaminofluorene, diethylnitrosamine, *m*-toluenediamine, and azo dyes (43–46). In mice, benzyl isothiocyanate blocked the neoplastic effects of diethylnitrosamine or benzo[*a*]pyrene on lung and forestomach (47, 48), and a variety of phenylalkyl isothiocyanates reduced the pulmonary carcinogenicity of

Table 2. Induction of QR and glutathione *S*-transferase (GST) in mouse tissues by sulforaphane and analogues

Inducer	Dose, μmol per mouse per day	Enzyme	Ratio of specific activities (treated/control)				
			Liver	Forestomach	Glandular stomach	Proximal small intestine	Lung
$\text{CH}_3\text{—S—(CH}_2\text{)}_4\text{—NCS}$ Erucin	15	QR	2.19 \pm 0.06	1.64 \pm 0.18*	1.72 \pm 0.11	3.10 \pm 0.20	1.66 \pm 0.13
		GST	1.86 \pm 0.08	2.51 \pm 0.11	2.07 \pm 0.08	3.00 \pm 0.21	1.41 \pm 0.11*
$\text{CH}_3\text{—S—(CH}_2\text{)}_3\text{—NCS}$ Sulforaphane	15	QR	2.45 \pm 0.07	1.70 \pm 0.18*	2.35 \pm 0.06	2.34 \pm 0.19	1.37 \pm 0.14*
		GST	1.86 \pm 0.08	1.98 \pm 0.08	2.97 \pm 0.08	2.13 \pm 0.20	1.17 \pm 0.09†
$\text{CH}_3\text{—S(=O)—(CH}_2\text{)}_4\text{—NCS}$ Erysolin	5	QR	1.62 \pm 0.09	1.05 \pm 0.21†	1.57 \pm 0.08†	1.22 \pm 0.20†	1.00 \pm 0.11†
		GST	1.08 \pm 0.11†	1.45 \pm 0.15†	1.94 \pm 0.10†	0.87 \pm 0.20†	1.09 \pm 0.13†

The compounds were administered to 6-week-old female CD-1 mice (4 or 5 mice per group) by gavage in indicated single daily doses in 0.1 ml of Emulphor EL 620P (GAF, Linden, NJ) for 5 days. Cytosols were prepared from the tissues 24 hr after the last treatment and assayed for enzyme activities (glutathione *S*-transferase was measured with 1-chloro-2,4-dinitrobenzene). The specific activities ($\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ \pm SEM) of organs of vehicle-treated control mice were as follows. Liver: QR, 47 \pm 0.70; GST, 1014 \pm 69. Forestomach: QR, 1038 \pm 155; GST, 1182 \pm 74. Glandular stomach: QR, 3274 \pm 85; GST, 1092 \pm 81. Small intestine: QR, 664 \pm 119; GST, 1572 \pm 266. Lung: QR, 54 \pm 5.8; GST, 439 \pm 34. Data are presented as mean \pm SEM. All ratios were significantly different from 1.0 with *P* < 0.01, except for *, *P* < 0.05, and †, *P* > 0.05.

the tobacco-derived carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (49, 50). The anticarcinogenic effects of previously studied isothiocyanates may be related to their capacity to induce phase II enzymes, which are involved in the metabolism of carcinogens (51-57).

It will be important to establish whether the alterations of drug metabolism observed in humans and rodents after the ingestion of cruciferous vegetables (58, 59) can be ascribed to their content of sulforaphane. The finding that this isothiocyanate is a major monofunctional inducer of phase II enzymes in broccoli also provides the possibility of clarifying the relationship between enzyme induction and chemoprotection.

We thank Andrew Ayer for supplying generous quantities of SAGA broccoli. These studies were supported by grants from the National Cancer Institute (PO1 CA 44530) and from the American Institute for Cancer Research. All animal experiments were in compliance with National Institutes of Health guidelines (60) and were approved by the Committee on Animal Care of Johns Hopkins University.

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General

Cancer preventive properties of varieties of *Brassica oleracea*: a review¹⁻³

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ABSTRACT Cabbage, broccoli, Brussels sprouts, and other members of the genus *Brassica* have been widely regarded as potentially cancer preventative. This view is often based on both experimental testing of crude extracts and epidemiological data. The experimental evidence that provides support for this possibility is reviewed for the commonly consumed varieties of *Brassica oleracea*. In a majority of cases the biological activities seen in testing crude extracts may be directly related to specific chemicals that have been reported to be isolated from one of these closely related species, thus the chemical evidence further supports the data from testing extracts and epidemiology. *Am J Clin Nutr* 1994;59(suppl):1166S-70S.

KEY WORDS *Brassica*, Brassicaceae, mutagen, antimutagen, cancer, prevention, vegetables, chemoprevention

Introduction

Although most botanists would hardly agree that "A rose may be a rose by any other name" there would be substantial agreement that a cabbage and a cauliflower may be quite the same. These vegetables, and other closely related members of the Brassicaceae family, have received widespread notice recently as public figures have disavowed their consumption and scientists have upheld them as exemplary of medicinally significant foods. Thus, in this article we review all of the experimental evidence that suggests that there may be a cancer preventive benefit from consumption of members of these closely related and commonly consumed vegetables (1, 2). Furthermore, in view of the extensive data (3, 4) that exist for these vegetables, we will restrict ourselves to those vegetables commonly classified as subvarieties of the species *Brassica oleracea* (Table 1).

From the outset it must be realized that the published experimental data come from two different types of experimental protocols. In the first type, evidence is published that concerns tests conducted on the whole food (or from crude extracts). In the second type, tests are conducted on specific chemical compounds that have been isolated from these foods. Specifically, we will cross-correlate these two bodies of data so that, whenever possible, the specific compounds that may be responsible for an observation seen in testing a crude extract are identified. It is worth noting that this information is often not available in the original article and lends credence to the initial observation.

It is our intention to provide support for observations made on crude extract and identify those areas in which the biologically active chemical species for a given observation may not yet be

identified. Although various aspects of the chemistry (5), pharmacology (6, 7), biology (8-10), and general concepts of cancer chemoprevention (11-13) have been reviewed separately, we will provide an overview approach that demonstrates the overlap between these various areas. Furthermore, it is important to recognize that many clinical trials are currently underway, (14) which, in preliminary reports, lend credence to the cancer preventative approaches (15, 16).

Relevant biological activities

The etiology of cancer follows no single track but rather is the result of an accumulation of diverse events that lead to a common endpoint, namely the uncontrolled growth of a normally quiescent cell. Nevertheless, there are generally recognized to be many common stages to the development of cancer. These stages (Fig 1) include an initial insult (or mutation) to the genetic material often delivered by a mutagen or other chemical agent but may also be inherited or possibly viral in origin. A cell that has received such an insult is said to be initiated. An initiated cell will still be quiescent and not manifest its altered phenotype until it is promoted. The promotional act may similarly take multiple forms but it fundamentally involves achieving a physiological state that signals the altered DNA to be read. Where the altered message leads to an unquenchable cycle of cellular division, the cell is considered cancerous. This aberrant equilibrium, where the cell cannot reset itself, will become a tumor if it cannot regain a "normal" or self-restrained equilibrium.

Cancer chemotherapeutic agents are directed against cancerous or fully promoted cells and seek to selectively kill the cell based on some aspect of its aberrant biochemical equilibrium. As such, all current cancer treatment is based on compounds that are toxic. An ideal cancer chemotherapeutic agent would be toxic only to cancer cells but the reality is that such specificity has not yet been achieved. Although this is clearly a suitable course when the fatality of the disease is considered, the approach to cancer

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² Supported in part by grant CA48112-01A3 from the National Cancer Institute.

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TABLE 1
The most commonly consumed members of the genus *Brassica*

Species	Variety	Common name
<i>Brassica campestris</i>		Field mustard
<i>Brassica chinensis</i>		Bok choy
<i>Brassica juncea</i>		Mustard greens
<i>Brassica napus</i>	var <i>napobrassica</i>	Rutabaga
<i>Brassica nigra</i>		Black mustard
<i>Brassica oleracea</i>	var <i>acephala</i>	Collards
<i>Brassica oleracea</i>	var <i>acephala</i>	Kale
<i>Brassica oleracea</i>	var <i>botrytis</i>	Broccoli
<i>Brassica oleracea</i>	var <i>botrytis</i>	Cauliflower
<i>Brassica oleracea</i>	var <i>capitata</i>	Cabbage
<i>Brassica oleracea</i>	var <i>gemmifera</i>	Brussels sprouts
<i>Brassica oleracea</i>	var <i>gongylodes</i>	Kohlrabi
<i>Brassica pekinensis</i>	var <i>capitata</i>	Cabbage (Chinese)
<i>Brassica rapa</i>	var <i>rapifera</i>	Turnip
<i>Brassica rapa</i>	var <i>japonica</i>	Red turnip

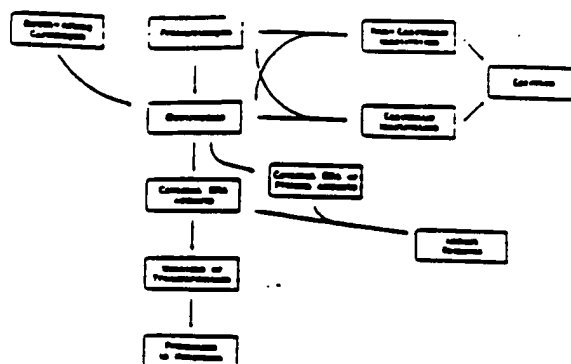


FIG 1. The etiology of cancer. The steps shown are those generally assumed in the development of cancer.

rather are of such a general nature that no specific mechanism of protection may be ascribed to them.

Antimutagenic activities

The ability of a crude extract of a *Brassica* variety to reduce the effect of a mutagen (either as a desmutagenic agent or as an antimutagenic agent) has been reported no less than eight times. In all of the cases in which a mechanism can be discerned it appears that, although the term antimutagen is used routinely, these are most likely all cases of desmutagenicity. These reports are summarized in Table 2.

The major bulk of the reports concern ability of a protein, termed the desmutagenic factor, to inhibit various mutagens in Ames-type assays. This factor, first described by Kada *et al* (24), was later characterized (25) and patented (26) by Morita *et al* as a heat-labile protein with a molecular weight of ~53 kDa, which contained a prosthetic group with a heme-like chromophore. This protein was shown active against tryptophan pyrolysates (24), ethidium bromide (25), 2-aminoanthracene (25), autooxidized linolenic acid (27), and pyrolysates for other amino acids (28).

TABLE 2
Summary of antimutagenic results

Plant extracted	Mutagen	Percent reduction
		%
Cauliflower	Nitrate + methyurea	78
Cauliflower	Nitrate + aminopyrine	57
Cabbage	Nitrate + sorbic acid	Moderate (not calculable)
Cauliflower	Nitrate + sorbic acid	Moderate (not calculable)
Cabbage	Tryptophan pyrolysate	97
Broccoli	Tryptophan pyrolysate-1	97
Broccoli	Tryptophan pyrolysate-2	81
Broccoli	Ethidium bromide	92
Broccoli	2-Aminoanthracene	84
Broccoli	AF-2	0
Broccoli	Oxidized linolenic acid	82
Cabbage	Oxidized linolenic acid	76
Red cabbage	Oxidized linolenic acid	81
Cauliflower	Oxidized linolenic acid	76
Cabbage	Tryptophan pyrolysate-2	35

chemoprevention must be based on a very different strategy. In view of the fact that such agents must be used prophylactically, they must exhibit few, if any, side effects and must have virtually no toxicity. In addition to these stringent requirements, it needs to be recognized that any compound that is to be considered as a cancer chemopreventive agent may also exhibit a suitable spectrum of biological activity.

In cancer chemoprevention the aim is to reduce the number of initiated cells, inhibit the promotion of initiated cells, or even reverse the promotion itself. Furthermore, each of these broad categories has many strategies that may be useful to cancer prevention. First, there are strategies that aim to reduce the initiation rate. The agents here are classified as antimutagens, desmutagens, inhibitors of enzymes that activate procarcinogens, or agents that stimulate the metabolism of mutagens to less harmful metabolites. Also included here are antioxidants because a portion of the genetic damage is likely the result of free radical damage (17-21). Second, there are strategies that aim to reverse or inhibit the promotion stages. Biological activities that act at this stage may act specifically on the promoted cell to cause it to redifferentiate and hence regain control of its own division or they may act at any of the points in one of the secondary messenger (or the related oncogene) systems that are frequently implicated as destabilizing agents. In the same light, the biological consequences of low-level inflammation or constant low-level estrogenic stimulation are similarly considered destabilizing (22, 23) and hence targets for chemopreventive approaches.

As we consider such an etiology, we can associate specific bioassays that have been described in the literature in relation to one or more of these points. Thus, as a basis for this article, we have undertaken to review the reports of relevant biological activities for *Brassica oleracea* varieties. They will be organized as discussed above. With respect to initiation stages, the majority of published literature in this area may be divided crudely into two broad groupings, namely reports of an anti- (or des-) mutagenic activity and reports of stimulation of a detoxification mechanism. With respect to antipromotion activity, there is a single report that suggests that this mechanism may play a role in *Brassica*'s cancer preventive potential. However, there are many reports that may not be classified as either of these stages but

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In their 1980 paper, Yamaguchi et al (27) demonstrated a striking correlation between the desmutagenic activity of the extracts and their peroxidase activity and further demonstrated that the peroxidase activity required a cofactor. This activity was later confirmed in the purified protein by Morita et al (25), who did not note the need for the cofactor. The signal characteristic to the desmutagenic factor has always been the fact that it is both heat labile and is inactivated by digestion with a proteinase. With this in mind, some workers (29) have pointed out that after heat treatment some crude extracts of *Brassica* extracts still exhibit residual activity, suggesting the presence of other antimutagenic components. Munzer (30) demonstrates that some antimutagenic activity acts by stimulating native detoxification systems in *Salmonella typhimurium* and thus some of these other agents are also desmutagenic.

The identity of the other antimutagenic agents has been the focus of other researchers. Two groups (31, 32) have reported that extracts of cauliflower and cabbage, respectively, interfere with the production of mutagens by nitrosation. There is considerable agreement that the active agents include ascorbic acid, cysteine, or other compounds acting as reducing agents. This is actually demonstrated by Osawa et al (32), who show that the ascorbic acid is responsible for the chemical reduction of the 1,2-dinitro-2-methyl pyrrole, the mutagenic nitrosation product of sorbic acid, to the nonmutagenic compound 1-nitro-2-methyl-4-amino pyrrole. Barale et al (31) show that ascorbic acid and some phenolic compounds can duplicate the activity seen in the crude extract. On the other hand, Lawson et al (33) have identified four specific compounds isolated from savoy chieftain cabbage that demonstrated antimutagenic activity against specific mutagens, *N*-methyl-*N*-nitrosourea (NMU) and 2-aminoanthracene (2-AA). These compounds, β -sitosterol, pheophytin-a, nonacosane, and nonacosanone, are notable because they are likely to be present in a majority of plants. These authors also demonstrate that commercial chlorophyll, the biological precursor to pheophytin-a, is strongly antimutagenic. These compounds were shown to present different activity profiles against the NMU and 2-AA; therefore, the authors argue that these compounds were achieving their antimutagenicity through more than one biological mechanism.

Stimulation of detoxification mechanisms

As noted briefly above, Munzer (30) noted that the antimutagenic activity of many vegetables, including cabbage, Brussels sprouts, and kohlrabi, was in stimulating the S-9 mix normally used to metabolize and sometimes activate mutagens. This observation serves to bridge the antimutagenic potential discussed above and the large body of data that makes it clear that in animals there is a strong stimulation of many of the native detoxification systems by extracts of various *Brassica* species. Although this attribute has been fairly widely discussed recently, because of the articles published by Talalay's group (34, 35), it is important to note that this area has a long and honorable background. Furthermore, although the Talalay articles do demonstrate a selectivity in the induction of phase-2 enzymes that has not previously reported, the ability of members of the Brassicaceae family to stimulate a broad spectrum of enzyme systems has been widely reported.

The earliest work on the induction of these enzyme systems was actually an attempt by Wattenberg (36) to explain variations

in baseline aryl hydrocarbon hydroxylase concentration, in different rat colonies. The variation ultimately was ascribed to the presence of alfalfa as an occasional component in rat chow. This observation was followed by an examination of the ability of many foods to stimulate this enzyme. Wattenberg and his group demonstrated that many members of the Brassicaceae family were also active in this regard (37) and, furthermore, the active compounds were readily identified as indole-3-carbinol, 3,3'-diindolymethane, and indole-3-acetonitrile, which stimulated 50-fold, 20-fold, and 6-fold increases, respectively, in enzyme activities in the livers of rats that consumed augmented basal chow. In subsequent papers they demonstrated that the ability of intestinal enzymes to detoxify many xenobiotic compounds, including the indoles noted above (38), correlated to Brussels sprouts or cabbage consumption in rats (39) and in humans (40). The enzyme systems involved included many mixed-function oxidases, such as phenacetin O-dealkylase, 7-ethoxycoumarin O-dealkylase, hexobarbital hydroxylase, and benzo(a)pyrene hydroxylase. A direct correlation was later established between the induction of these activities and the concentration of these compounds by McDanell et al (41, 42). These later studies also demonstrated that the various active compounds had differing abilities to stimulate enzymes in different organs of the body. They note for instance that the ascorbic acid conjugate of indole-3-carbinol is the most active compound in stimulating the mixed-function oxidase populations of the gut whereas indole-3-carbinol, of the compounds tested, was the strongest inducer of the liver enzymes. Tanaka et al (43) demonstrated recently the ability of indole-3-carbinol to inhibit tongue carcinogenesis induced with 4-nitroquinoline-1-oxide.

Meanwhile, working in a parallel vein, Salbe and Bjeldanes (44) not only confirmed the earlier results of the Wattenberg group but also demonstrated that the enzyme glutathione-S-transferase was also strongly induced by Brussels sprouts. This enzyme, unlike those discussed earlier, is not a P-450 type enzyme but represents rather a phase-2 detoxification system that acts to conjugate and clear toxicants from the system. The significance of this difference cannot be understated. For most of the P-450 type enzymes, their ability to detoxify many mutagens must always be balanced by their ability to activate other mutagens (45). For glutathione-S-transferase, there are no such drawbacks; rather, as this group has shown (46), an increase in this enzyme alone directly resulted in an 87% reduction in the binding of aflatoxin to hepatic DNA in vivo. A wide spectrum of compounds (47, 48) including the glucosinolates, such as sinigrin and progoitrin, and their derivatives, such as allyl isothiocyanate, goitrin, indole-3-carbinol, and indole-acetonitrile, induce glutathione-S-transferase. In other systems it is induced even more strongly by xanthotoxin and some flavonoids (49).

Other relevant reports

There are some reports in which no mechanism can be easily ascribed to the results or that do not fit into either of the above two categories. These reports are nonetheless potentially significant with respect to the ability of brassicaceous plants to be cancer chemopreventive. The first of these concerns a study conducted by Bresnick et al (50) in which rats were fed a controlled fat diet with and without cabbage. There was found to be a statistically significant reduction in the rate of chemically induced

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breast tumors in the rats with cabbage in their diet. This effect was not seen in rats on a high-fat diet. It is of interest to note that the experimental design allowed for the consumption of cabbage only after the initiation event, administration of MNU, thus indicating a potential antipromotion effect. This possibility is also suggested by a report from Koshimizu et al (51), who use the inhibition of Epstein-Barr virus induction as an indication of antipromotion activity. In their assay an extract of cauliflower is very strongly active in inhibiting the normal promotion event. In neither of these publications is it possible to ascribe a specific compound to the activity observed.

Finally, note that protease inhibitors have been associated with carcinogenesis inhibition (52, 53), so the relevance of a strong trypsin inhibitor from the seed of kale (*Brassica oleracea var sabellica*) may be relevant (54). The presence of this agent in other parts of the plant (much less in other varieties) or its ability to overcome problems of absorption and transport are totally unknown.

Summary

It may at first seem surprising that so many biological activities have been demonstrated for plants as commonly consumed as these. Yet reflection on the complex chemical nature of most plants suggests that there may be more biological potential in all of them than we would expect from something that is generally considered to be biologically neutral. Furthermore, although some of these reports have been in humans, the majority are in vitro results whose bearing on their effect on humans is very much an open question. The work of McDaniel et al (41, 42) clearly demonstrates the importance of transport and the variable ability of different metabolites of even the same compound to affect different organs. A report by Birt et al (55) amplifies this by demonstrating that although the effect of cabbage is beneficial in some cases it may act to increase tumorigenicity in other model systems (or cancer types).

We have presented a case that strongly implies that the cancer preventive potential of many members of the Brassicaceae family is strong, yet it must always be stressed that to understand the relevance of these reports on the human condition, many further studies need to be done to specifically address questions of the stability, bioavailability, transport, and metabolism. The additive or even synergistic effects of these compounds are unknown. The additional effects of normal food preparation procedures present another factor that is yet largely unexplored with respect to the cancer preventive properties. In brief, there is much exciting potential in the cancer preventive properties and yet there is, as of this writing, no absolute statement that can be made concerning the ability of these foods to directly alter the course of carcinogenesis.

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L Number	Hits	Search Text	DB	Time stamp
1	128	glucosinolate\$2	USPAT; US-PGPUB	2002/05/01 21:14
2	13732	isothiocyanate\$2	USPAT; US-PGPUB	2002/05/01 21:15
3	45	glucosinolate\$2 and isothiocyanate\$2	USPAT; US-PGPUB	2002/05/01 21:15
5	6	((glucosinolate\$2 and isothiocyanate\$2) and plant\$2 and (extract\$7 or isolat\$6) and homogen\$) and (di\$2methyl adj sulfoxide\$2 or di\$2methylsulfoxide\$2) and acetonitrile\$2 and di\$2methylformamide	USPAT; US-PGPUB	2002/05/01 21:19
4	26	(glucosinolate\$2 and isothiocyanate\$2) and plant\$2 and (extract\$7 or isolat\$6) and homogen\$	USPAT; US-PGPUB	2002/05/01 21:27
6	16	(glucosinolate\$2 and isothiocyanate\$2) and plant\$2 and (extract\$7 or isolat\$6) and homogen\$ and solvent\$2	USPAT; US-PGPUB	2002/05/01 21:27



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/825,989	04/05/2001	Jed W. Fahey	046585/0138	4463

7590

05/22/2002

Richard C. Peet
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 Washington Harbour
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EXAMINER

DELACROIX MUIRHEI, CYBILLE

ART UNIT

PAPER NUMBER

1614

DATE MAILED: 05/22/2002

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary

Application No.

09/825,989

Applicant(s)

FAHEY ET AL.

Examiner

Cybille Delacroix-Muirheid

Art Unit

1614

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 05 April 2001.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 48-67 is/are pending in the application.
- 4a) Of the above claim(s) _____ is/are withdrawn from consideration.
- 5) ☒ Claim(s) 48-52 and 54-57 is/are allowed.
- 6) ☒ Claim(s) 58-63 is/are rejected.
- 7) ☒ Claim(s) 53 and 64-67 is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
- 11) ☐ The proposed drawing correction filed on _____ is: a) ☐ approved b) ☐ disapproved by the Examiner.
If approved, corrected drawings are required in reply to this Office action.
- 12) ☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

- 13) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
- * See the attached detailed Office action for a list of the certified copies not received.
- 14) ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).
a) ☐ The translation of the foreign language provisional application has been received.
- 15) ☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

- | | |
|--|---|
| 1) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892) | 4) <input type="checkbox"/> Interview Summary (PTO-413) Paper No(s). _____ |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948) | 5) <input type="checkbox"/> Notice of Informal Patent Application (PTO-152) |
| 3) <input checked="" type="checkbox"/> Information Disclosure Statement(s) (PTO-1449) Paper No(s) <u>3</u> . | 6) <input type="checkbox"/> Other: |

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DETAILED ACTION

The following is responsive to the preliminary amendment received April 5, 2001.

Claims 1-47 are cancelled without prejudice or disclaimer. New claims 48-67 are added.

Claims 48-67 are presented for prosecution on the merits.

Information Disclosure Statement

Applicant's Information Disclosure Statement received April 5, 2001 has been considered in part, i.e. US patents. The remaining articles A3-A76 were not in parent application 09/425,890. The Examiner respectfully requests that Applicant submit these references so that they may be considered and made of record.

Claim Objections

1. Claim 53 is objected to because of the following informalities: in claim 53, lines 1-3, selected from the group consisting of...or is improper Markush terminology. The "or" at line 3, before "plant parts" should be deleted and replaced with --and--. Please see MPEP 2173.05(h). Appropriate correction is required.

Claim Rejections - 35 USC § 112

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2. Claims 59, 61, 62 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

3. Claims 59, 61, 62 recite the limitation "said solvent" in line 1. There is insufficient antecedent basis for this limitation in the claim. It is respectfully requested that Applicant amend claims 59, 61, 62 to add the term --non-toxic-- before "solvent."

Allowable Subject Matter

Claims 48-57 are free from the prior art because the prior art does not disclose or fairly suggest Applicant's claimed method.

Claim Rejections - 35 USC § 102

4. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless -

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

5. Claims 58, 59, 62, 63 are rejected under 35 U.S.C. 102(b) as being anticipated by Jones et al., 4,158,656.

Jones et al. disclose a method for extracting glucosinolates, the method comprising contacting seed material (rapeseed) with an aqueous-lower alkanol (water-alcohol, i.e. ethanol)solvent

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solution at a temperature below 60° C and under conditions so as to prevent enzymatic degradation of the glucosinolates. Jones et al. additionally disclose that the temperature is kept below 60° C in order to prevent activation of the myrosinase. Please see claim 1; col. 1, lines 3-6; col. 4, lines 44-63.

6. Claims 58, 59, 63 are rejected under 35 U.S.C. 102(b) as being anticipated by Anjou et al., 4,083,836.

Anjou et al. teach a method for extracting or leaching glucosinolates from seed material, the method comprising obtaining a meat fraction of the seed material and subjecting the meat fraction to a wet state at 80-100°C and leaching the glucosinolates by water, wherein the temperature of the leaching is 60-80°C. Please see the abstract; col. 5, lines 21-57.

Please note that Applicant's claims recite "comprising" language. The transitional term "comprising", which is synonymous with "including," "containing," or "characterized by," is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. See, e.g., *Genentech, Inc. v. Chiron Corp.*, 112 F.3d 495, 501, 42 USPQ2d 1608, 1613 (Fed. Cir. 1997) ("Comprising" is a term of art used in claim language which means that the named elements are essential, but other elements may be added and still form a construct within the scope of the claim.); *Moleculon Research Corp. v. CBS, Inc.*, 793 F.2d 1261, 229 USPQ 805 (Fed. Cir. 1986); *In re Baxter*, 656 F.2d 679, 686, 210 USPQ 795, 803 (CCPA 1981); *Ex parte Davis*, 80USPQ 448, 450 (Bd. App. 1948) ("comprising" leaves "the claim open for the inclusion of unspecified ingredients even in major amounts").

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7. Claims 61 and 64-67 are objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

Claim Rejections - 35 USC § 103

8. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

9. The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any

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evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103© and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

10. Claim 60 is rejected under 35 U.S.C. 103(a) as being unpatentable over Anjou et al., supra.

Anjou et al. as applied above.

Anjou et al. does not disclose that the temperature of the leach water is 100°C; however, since Anjou et al. establish that the glucosinolate leaching process is temperature dependent, it would have been obvious to one of ordinary skill in the art at the time the invention was made to further modify the leaching method of Anjou et al. such that the temperature is effective to result in optimum extraction of glucosinolates from the seed material. Such a modification would have been motivated by the reasoned expectation of successfully extracting glucosinolates from the seed material.

Conclusion

Claims 58, 59, 60, 61, 62, 63 are rejected.

Claims 53, 64-67 are objected to.

Claims 48-57 are free from the prior art.

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Any inquiry concerning this communication or earlier communications from the examiner should be directed to Cybille Delacroix-Muirheid whose telephone number is (703) 306-3227. The examiner can normally be reached on Tue-Fri from 8:30 to 6:00. The examiner can also be reached on alternate Mondays.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Marianne Seidel, can be reached on (703) 308-4725. The fax phone number for this Group is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-1235.

CDM



May 16, 2002


Cybille Delacroix-Muirheid
Patent Examiner Group 1600

Notice of References Cited

Application/Control No.

09/825,989

Applicant(s)/Patent Under
Reexamination
FAHEY ET AL.

Examiner

Cybille Delacroix-Muirheid

Art Unit

1614

Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-4,083,836	04-1978	ANJOU et al.	260/123.5
	B	US-4,158,656	06-1979	JONES et al.	260/123.5
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	<i>cdm</i> <i>5/16/02</i>

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
 Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Form PTO-1449 (MODIFIED)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. 046585/0138	SERIAL NO. <u>09/825,989</u> <u>Unassigned</u>
INFORMATION DISCLOSURE CITATION (Use several sheets if necessary)		APPLICANT Jed FAHEY et al.	
		FILING DATE April 5, 2001	GROUP ART UNIT <u>1614</u> <u>Unassigned</u>

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	REF	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB- CLASS	FILING DATE IF APPROPRIATE
<i>cm</i>	A1	5,725,895	3/98	Fahey et al.	426	49	
<i>cm</i>	A2	4,511,988 5,411,986	5/95 5/95	Cho et al.	514	514	

FOREIGN PATENT DOCUMENTS

	REF	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB- CLASS	TRANSLATION	
							YES	NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	A3	The Sproutletter, Number 25, Nov. – Dec. 1984.
	A4	"The Sproutletter" May-June 1981, No. 4.
	A5	Roy Bruder, Ph.D., Discovering Natural Foods, (including pgs.203-209), Woodbridge Press, 1982.
	A6	Brian R. Clement, Hippocrates Health Program, (including pgs 7-11), Hippocrates Publications, 1989.
	A7	Jethro Kloss, The Back to Eden Cookbook, pgs. 61-61, Woodbridge Press, 1974.

EXAMINER <i>cm</i>	DATE CONSIDERED <i>5-15-02</i>
* EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include any copy of this form with next communication to applicant.	

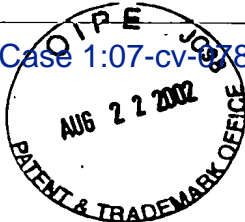
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				FILING DATE April 5, 2001		GROUP ART UNIT Unassigned	
OTHER DOCUMENTS <i>(Including Author, Title, Date, Pertinent Pages, Etc.)</i>							
	A8	Steve Meyerowitz, Sproutmann Kitchen Garden Cookbook, The Sprouthouse, Inc., pgs. 178-179, 290, 1994.					
	A9	Steve Meyerowitz, Sprout It, One week from Seed to Salad, The Sprouthouse, Inc., (including pgs. 84-85, 120-123), June 1994.					
	A10	Steve Meyerowitz, The Complete Guide to Sprouting, Sprouts The Miracle Food, Sproutman Publications, (including pgs. 121-2), May 1998.					
	A11	Esther Munroe, Sprouts to Grow and Eat, (including pgs. 2-15), Dec. 1974.					
	A12	Jean Hewitt, The New York Times "New Natural Foods Cookbook", Avon Books, pgs. 200-203, 1982.					
	A13	Martha H. Oliver, Add a Few Sprouts To Eat Better for Less Money, Pivot Original Health Books, (including pgs. 52-53, 118-119), 1975.					
	A14	James C. Schmidt, Horticulture Facts, "Growing Sprouts Indoors", (Rev. 4/81).					
	A15	Angnes Toms, The Joy of Eating Natural Foods, The Complete Organic Cookbook, pgs. 318-319, Nov. 1971.					
	A16	Karen Cross Whyte, The Complete Sprouting Cookbook, Troubador Press, (including pgs. 57-59), 1973.					
	A17	Ann Wigmore, The Sprouting Book, Avery Publications, (including pgs. 29-37), 1986.					
	A18	Debra Schwarze, Growing Sprouts, Neb Guide, Jan. 1989.					
	A19	John Tobe, Sprouts Elixir of Life", 1970.					
	A20	Alicia Bay Laurel, "Living on the Earth" a Vintage Book.					
	A21	David Ehrlich with George Wolf, Foreward by Peter Albright, M.D., "The Bowell Book", Schocken Books, 1981.					
	A22	"The Good News Sprouts Recipe Book" ISGA, Aug. 1992.					

Form PTO-1449 (MODIFIED)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTY. DOCKET NO. 046585/0138		SERIAL NO. Unassigned	
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OTHER DOCUMENTS <i>(Including Author, Title, Date, Pertinent Pages, Etc.)</i>							
	A23	Ann Wigmore, "The Hippocrates Diet and Health Program", Avery Publications, 1984.					
	A24	Sprouting Publications Oahspe Foundation, Health and Sprouting Supplies.					
	A25	Sproutletter, #41, Summer, 1989.					
	A26	The Sproutletter, Number 27, March-April 1985.					
	A27	Steve Meyerowitz, Growing Vegetables Indoors", 1983.					
	A28	The Sproutletter, Number 24, Sept.-Oct. 1984.					
	A29	The Sproutletter, Issue 33, Spring 1987.					
	A30	The Sproutletter, Number 28, May-June 1985.					
	A31	The Sproutletter, Number 26, Jan-Feb 1985.					
	A32	Sprouting Publications, Health and Sprouting Supplies.					
	A33	The Sproutletter, Number 29, July – August 1985.					
	A34	Sproutletter, #40, Spring, 1989.					
	A35	The Sproutletter, Number 32, Summer.					
	A36	Sproutletter, #44, March 1991.					
	A37	Sproutletter, #36, Winter, 1987-88.					

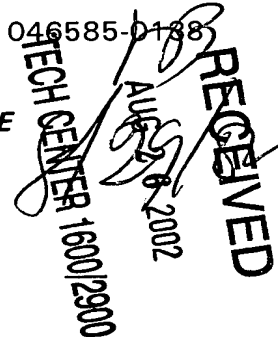
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OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)							
	A38	Sproutletter, #39, Fall, 1988.					
	A39	Sproutletter, #43, May/June 1990.					
	A40	Sproutletter, #38, Summer, 1988.					
	A41	Sprouting Publications Health and Sprouting Supplies.					
	A42	Spring Sale for Members Only.					
	A43	The Sproutletter, A newsletter of useful and unusual information on sprouts, raw foods and nutrition.					
	A44	The Sproutletter, #31, Winter.					
	A45	Deirdre Purdy, ed., The Summer Kitchen, A Farmers' Market Cookbook, 1981.					
	A46	Viktoras Kulvinskas, M.S. Co-Director Hippocrates Health Institute, "Love Your Body or how to be a live food lover", 1974.					
	A47	The Sprout House Article from Newspaper.					
	A48	New Prices – New Products, July 1985 order form.					
	A49	Steve Meyerowitz, Indoor Vegetable Kit, The Sprout House.					
	A50	The Sprout House Newsletter, Issue #15, August, 1992.					
	A51	Sproutman's Exotic Seeds for Sprouting 100% Organically Grown Order Form.					
	A52	Complaint for Patent Infringement (Brassica Protection Products, LLC v. The Sproutman, Inc. dated September 20, 1999.					

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OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)							
	A53	Murry Tizer's Answer, Affirmative Defenses and Counterclaims dated June 28, 1999					
	A54	The Sproutman, Inc.'s Answer, Affirmative Defenses and Counterclaims dated June 28, 1999					
	A55	Request for Reexamination of U.S. Patent No. 5,725,895 filed October 11, 1999					
	A56	Sprout it! One Week From Seed to Salad, Steve Meyerowitz (The Sprout House, Inc., Great Barrington, MA),. Pages 20-21, 58, 85-86, 120-123, 1983					
	A57	Munroe, E., Sprouts to Grow and Eat, the Steven Greene Press, (1974), pp. 2-9 and 14-15					
	A58	Schmidt, James C., Growing Sprouts Indoors, Cooperative Extension Service of the University of Illinois at Urbana-Champaign, College of Agriculture (1984) (pamphlet)					
	A59	Whyta, K.C., The Complete Sprouting Cookbook, Troubador Press (1983), pp 10-16, 57-60					
	A60	The Sprout House cookbook, Steve Meyerowitz (The Sprout House, Inc. Great Barrington, MA 1983), pages 20-21, 85, 120-123.					
	A61	The Good New Sprouts Recipe Book, International Sprout Growers Association, pp. 1-8, August 1992.					
	A62	Posner et al., "Design and Synthesis...and potency as Inducers of Anticarcinogenic Detoxication Enzymes", Journal of Medicinal Chemistry, Vol. 37, No. 1, pp. 170-175, 1994					
	A63	Zhang et al., "A major inducer of Anticarcinogenic protective enzymes from broccoli; isolation And education of structure", Proc. Natl. Acad. Sci. USA, Vol. 89, pp. 2399-2403, March 1992.					
	A64	Prochaska et al. "Rapid detective of inducers of enzymes that protect against carcinogens", Proc. Nat'l. Sci. USA Vol. 89, pp. 2394-2388, March 1992					
	A65	Zhang et al., "Anticarcinogenic activities of sulforaphane and structurally related synthetic norbomyl isothiocyanates", Proc. Natl. Sci. USA, Vol. 91, pp. 3147-3150, April 1994.					
	A66	Prochaska et al., "Regulatory Mechanisms of Monofunctional and Bifunctional Anticarcinogenic Enzyme, Inducers in Murine Liver,,: Cancer Research Vol. 48, pp. 4776-4782, September 1988.					

Form PTO-1449 (MODIFIED)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTY. DOCKET NO. 046585/0138		SERIAL NO. Unassigned	
INFORMATION DISCLOSURE CITATION (Use several sheets if necessary)				APPLICANT Jed FAHEY et al.			
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OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)							
	A67	Prochaska et al., "Direct Measurement of NAD(P)H: Quinone Reductase from Cells Cultured in Microtiter Wells: A Screening Assay for...Inducers", analytical Biochemistry, Vol. 169, pp. 328-336, 1988.					
	A68	Beecher, "Cancer prevention properties of varieties of Brassica oleracea: a review 1-3 ", Am J. Clin. Nutr.: 59 (suppl.) pp. 1166 s-1170s, 1994.					
	A69	Presters et al., "Chemical and molecular regulation of enzymes that detoxify carcinogens", Proc. Natl. Acad. Sci. USA, Vol. 90, pp. 2965-2969, April 1993.					
	A70	Zhand et al., "Anticarcinogenic Activities of Organic Isothiocyanates: Chemistry and Mechanisms", Cancer Research suppl., 54, pp. 1976s-1981s, April 1, 1994.					
	A71	Talay, "The role of Enzyme Induction in Protection Against Carcinogenesis", Cancer Chemoprevention, pp. 469-478, 1992.					
	A72	Prester et al., "The Electrophile Counterattack Response: Protection Against Neoplasia and Toxicity", Advan. Enzyme Regul., vol. 33, pp. 281-296, 1993.					
	A73	Masilungan et al., "Screening of Philippine Medicinal Plants for Anticancer Agents using CCNSC "Protocols", Cancer Chemotherapy Reports (Part 2) Vol. 2, No.1, pp. 135-140, April 1971.					
	A74	Polasa et al., "Cancer preventive properties of varieties of Brassica oleracea: A review Source", American Journal of Clinical Nutrition 59 (5 Suppl), 1994.					
	A75	Patent Abstract of Japan Sect. No. 305, Vol. 9, No. 2371, p.2, September 1985.					
	A76	Barrett et al., "Protective Effect of Cruciferous Seed Meals Against Mouse Colon Cancer", Cereal Foods World 613, Vol. 39, No. 8, pp. 613, August 1994.					
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Atty. Dkt. No. 046585-0138



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Jed FAHEY et al.

Title: CANCER CHEMOPROTECTIVE FOOD PRODUCTS

Appl. No.: 09/825,989

Filing Date: 04/05/2001

Examiner: Cybille Delacroix-Muirhei

Art Unit: 1614

INFORMATION DISCLOSURE STATEMENT
UNDER 37 CFR §1.56

Commissioner for Patents
Washington, D.C. 20231

Sir:

Submitted herewith on Form PTO/SB/08 is a listing of documents known to Applicants in order to comply with Applicants' duty of disclosure pursuant to 37 CFR §1.56. A copy of each listed document is being submitted to comply with the provisions of 37 CFR §1.97 and §1.98.

The submission of any document herewith, which is not a statutory bar, is not intended as an admission that such document constitutes prior art against the claims of the present application or that such document is considered material to patentability as defined in 37 CFR §1.56(b). Applicants do not waive any rights to take any action which would be appropriate to antedate or otherwise remove as a competent reference any document which is determined to be a *prima facie* art reference against the claims of the present application.

TIMING OF THE DISCLOSURE

The listed documents are being submitted in compliance with 37 CFR §1.97(c), before the mailing date of either a final action under 37 CFR §1.113, a notice of allowance under 37 CFR §1.113, or an action that otherwise closes prosecution in the application.

RELEVANCE OF EACH DOCUMENT

All of the documents are in English.

Applicants respectfully request that any listed document be considered by the Examiner and be made of record in the present application and that an initialed copy of Form PTO/SB/08 be returned in accordance with MPEP §609.

STATEMENT

The undersigned hereby states in accordance with 37 CFR §1.97(e)(1) that each item of information contained in this information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three (3) months prior to filing of this Statement.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 CFR §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741.

Atty. Dkt. No. 046585-0138

Respectfully submitted,

Date August 22, 2002

By Richard C. Peet

FOLEY & LARDNER
Customer Number: 22428



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Richard C. Peet
Attorney for Applicant
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046585/0138

SERIAL NO.

Unassigned

APPLICANT

Jed FAHEY et al.

FILING DATE

April 5, 2001

GROUP ART UNIT

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INFORMATION DISCLOSURE CITATION

(Use several sheets if necessary)

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	REF	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB- CLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

REF	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB- CLASS	TRANSLATION	
						YES	NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

A3	The Sproutletter, Number 25, Nov. – Dec. 1984.
A4	"The Sproutletter" May-June 1981, No. 4.
A5	Roy Bruder, Ph.D., Discovering Natural Foods, (including pgs.203-209), Woodbridge Press, 1982.
A6	Brian R. Clement, Hippocrates Health Program, (including pgs 7-11), Hippocrates Publications, 1989.
A7	Jethro Kloss, The Back to Eden Cookbook, pgs. 61-61, Woodbridge Press, 1974.

EXAMINER

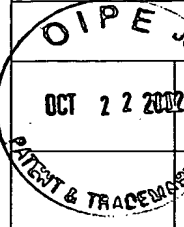
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A8	Steve Meyerowitz, Sproutmann Kitchen Garden Cookbook, The Sprouthouse, Inc., pgs. 178-179, 290, 1994.		
A9	Steve Meyerowitz, Sprout It, One week from Seed to Salad, The Sprouthouse, Inc., (including pgs. 84-85, 120-123), June 1994.		
A10	Steve Meyerowitz, The Complete Guide to Sprouting, Sprouts The Miracle Food, Sproutman Publications, (including pgs. 121-2), May 1998.		
A11	Esther Munroe, Sprouts to Grow and Eat, (including pgs. 2-15), Dec. 1974.		
A12	Jean Hewitt, The New York Times "New Natural Foods Cookbook", Avon Books, pgs. 200-203, 1982.		
A13	Martha H. Oliver, Add a Few Sprouts To Eat Better for Less Money, Pivot Original Health Books, (including pgs. 52-53, 118-119), 1975.		
A14	James C. Schmidt, Horticulture Facts, "Growing Sprouts Indoors", (Rev. 4/81).		
A15	Angnes Toms, The Joy of Eating Natural Foods, The Complete Organic Cookbook, pgs. 318-319, Nov. 1971.		
A16	Karen Cross Whyte, The Complete Sprouting Cookbook, Troubador Press, (including pgs. 57-59), 1973.		
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A18	Debra Schwarze, Growing Sprouts, Neb Guide, Jan. 1989.		
A19	John Tobe, Sprouts Elixir of Life", 1970.		
A20	Alicia Bay Laurel, "Living on the Earth" a Vintage Book.		
A21	David Ehrlich with George Wolf, Foreward by Peter Albright, M.D., "The Howell Book", Schocken Books, 1981.		
A22	"The Good News Sprouts Recipe Book" ISGA, Aug. 1992.		

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	A23	Ann Wigmore, "The Hippocrates Diet and Health Program", Avery Publications, 1984.			
	A24	Sprouting Publications Oahspe Foundation, Health and Sprouting Supplies.			
	A25	Sproutletter, #41, Summer, 1989.			
	A26	The Sproutletter, Number 27, March-April 1985.			
	A27	Steve Meyerowitz, Growing Vegetables Indoors", 1983.			
	A28	The Sproutletter, Number 24, Sept.-Oct. 1984.			
	A29	The Sproutletter, Issue 33, Spring 1987.			
	A30	The Sproutletter, Number 28, May-June 1985.			
	A31	The Sproutletter, Number 26, Jan-Feb 1985.			
	A32	Sprouting Publications, Health and Sprouting Supplies.			
	A33	The Sproutletter, Number 29, July – August 1985.			
	A34	Sproutletter, #40, Spring, 1989.			
	A35	The Sproutletter, Number 32, Summer.			
	A36	Sproutletter, #44, March 1991.			
	A37	Sproutletter, #36, Winter, 1987-88.			

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A38	Sproutletter, #39, Fall, 1988.		
A39	Sproutletter, #43, May/June 1990.		
A40	Sproutletter, #38, Summer, 1988.		
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A42	Spring Sale for Members Only.		
A43	The Sproutletter, A newsletter of useful and unusual information on sprouts, raw foods and nutrition.		
A44	The Sproutletter, #31, Winter.		
A45	Deirdre Purdy, ed., The Summer Kitchen, A Farmers' Market Cookbook, 1981.		
A46	Viktoras Kulvinskas, M.S. Co-Director Hippocrates Health Institute, "Love Your Body or how to be a live food lover", 1974.		
A47	The Sprout House Article from Newspaper.		
A48	New Prices – New Products, July 1985 order form.		
A49	Steve Meyerowitz, Indoor Vegetable Kit, The Sprout House.		
A50	The Sprout House Newsletter, Issue #15, August, 1992.		
A51	Sproutman's Exotic Seeds for Sprouting 100% Organically Grown Order Form.		
A52	Complaint for Patent Infringement (Brassica Protection Products, LLC v. The Sproutman, Inc. dated September 20, 1999.		

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OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)			
	A53	Murry Tizer's Answer, Affirmative Defenses and Counterclaims dated June 28, 1999	
	A54	The Sproutman, Inc.'s Answer, Affirmative Defenses and Counterclaims dated June 28, 1999	
	A55	Request for Reexamination of U.S. Patent No. 5,725,895 filed October 11, 1999	
	A56	Sprout it! One Week From Seed to Salad, Steve Meyerowitz (The Sprout House, Inc., Great Barrington, MA),. Pages 20-21, 58, 85-86, 120-123, 1993.	
	A57	Munroe, E., Sprouts to Grow and Eat, the Steven Greene Press, (1974), pp. 2-9 and 14-15	
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	Beecher, "Cancer prevention properties of varieties of Brassica oleracea: a review" ^{1-3 *} , Am J. Clin. Nutr.: 59 (suppl.) pp. 1166 s-1170s, 1994.						
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	Zhand et al., "Anticarcinogenic Activities of Organic Isothiocyanates: Chemistry and Mechanisms", Cancer Research suppl., 54, pp. 1976s-1981s, April 1, 1994.						
A69	Talalay, "The role of Enzyme Induction in Protection Against Carcinogenesis", Cancer Chemoprevention, pp. 469-478, 1992.						
	Prester et al., "The Electrophile Counterattack Response: Protection Against Neoplasia and Toxicity", Advan. Enzyme Regul., vol. 33, pp. 281-296, 1993.						
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	Polasa et al., "Cancer preventive properties of varieties of Brassica oleracea: A review Source", American Journal of Clinical Nutrition 59 (5 Suppl), 1994.						
A71	Patent Abstract of Japan Sect. No. 305, Vol. 9, No. 2371, p.2, September 1985.						
	Barrett et al., "Protective Effect of Cruciferous Seed Meals Against Mouse Colon Cancer", Cereal Foods World 613, Vol. 39, No. 8, pp. 613, August 1994, Abstract #127.						
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A01N 47/40, C07C 331/04, 331/12	A1	(11) International Publication Number: WO 94/19948 (43) International Publication Date: 15 September 1994 (15.09.94)
(21) International Application Number: PCT/US94/02453 (22) International Filing Date: 11 March 1994 (11.03.94) (30) Priority Data: 030,610 12 March 1993 (12.03.93) US (71)(72) Applicants and Inventors: CHO, Cheon-Gyu [KR/US]; 110 West 39th Street, Baltimore, MD 21210 (US). POS- NER, Gary, H. [US/US]; 3216 Timberfield Lane, Baltimore, MD 21208 (US). TALALAY, Paul [US/US]; 5512 Box- hill Lane, Baltimore, MD 21212 (US). ZHANG, Yuesheng [CN/US]; 105 Est 39th Street, Baltimore, MD 21210 (US). (74) Agents: HOSCHEIT, Dale, H. et al.; Banner, Birch, McKie & Beckett, Suite 1100, 1001 G Street, N.W., Washington, DC 20001-4597 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: CHEMOPROTECTIVE ISOTHIOCYANATES (57) Abstract <p>Sulforaphane has been isolated and identified as a major and very potent phase II enzyme inducer in broccoli (<i>Brassica oleracea italica</i>). Sulforaphane is a monofunctional inducer, inducing phase II enzymes selectively without the induction of aryl hydrocarbon receptor-dependent cytochromes P-450 (phase I enzymes). Analogues differing in the oxidation state of sulfur and the number of methylene groups were synthesized, and their inducer potencies were measured. Sulforaphane is the most potent of these analogues. Other analogues having different substituent groups in place of the methylsulfinyl group of sulforaphane were also synthesized and assessed. Of these, the most potent are 6-isothiocyanato-2-hexanone and <i>exo</i>-2-acetyl-6-isothiocyanatonorbomane.</p>		

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CHEMOPROTECTIVE ISOTHIOCYANATES

This invention was made with support from the National Institutes of Health, Grant No. CA44530. The U.S. government therefore retains certain rights in the invention.

TECHNICAL FIELD OF THE INVENTION

This invention relates to compounds which stimulate mammalian enzymes which are involved in detoxication of carcinogens. More specifically, it relates to compounds which induce the activity of quinone reductase¹, glutathione transferases and other phase II enzymes, without inducing the activity of cytochromes P-450.

BACKGROUND OF THE INVENTION

Extrinsic factors, including personal life-styles, play a major role in the development of most human malignancies (Wynder, et al., *J. Natl. Cancer Inst.* 58:825-832 (1977); Higginson, et al., *J. Natl. Cancer Inst.* 63:1291-1298 (1979); Doll, et al., *J. Natl. Cancer Inst.* 63:1191-1308 (1981)). Cigarette smoking and

¹Abbreviation: QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2].

consumption of alcohol, exposure to synthetic and naturally occurring carcinogens, radiation, drugs, infectious agents, and reproductive and behavioral practices are now widely recognized as important contributors to the etiology of cancer. But perhaps most surprising is the inference that normal human diets play causative roles in more than one-third (and possibly even two-thirds) of human neoplasia (Wynder, et al., *J. Natl. Cancer Inst.* 58:825-832 (1977); Higginson, et al., *J. Natl. Cancer Inst.* 63:1291-1298 (1979); Doll, et al., *J. Natl. Cancer Inst.* 63:1191-1308 (1981)). Our food contains not only numerous mutagens and carcinogens but also a variety of chemicals that block carcinogenesis in animal models (Ames, *Science* 221:1256-1264 (1983); Ames, et al. *Proc. Natl. Acad. Sci. USA* 87:7777-7781 (1990); Ames, et al., *Proc. Natl. Acad. Sci. USA* 87:7782-7786 (1990); Carr, B.I., *Cancer* 55:218-224 (1985); Fiala, et al., *Annu. Rev. Nutr.* 5:295-321 (1985); Wattenberg, *Cancer Res. Suppl.* 43:2448s-2453s (1983); Wattenberg, *Cancer Res.* 45:1-8 (1985); Wattenberg, et al., *Diet, Nutrition and Cancer*:193-203 (1986)). Furthermore, carcinogens can even protect against their own toxic and neoplastic effects or those of other carcinogens – i.e., carcinogens may act as anticarcinogens (Richardson, et al., *Cancer Res.* 11:274 (1951); Huggins, et al., *J. Exp. Med.* 119:923-942 (1964); Huggins, et al., *J. Exp. Med.* 119:943-954 (1964)).

Clearly, dietary modifications modulate cancer risk in various ways: for instance, through changes in caloric intake, by altering the consumption of nutritive and nonnutritive major components, and by providing exposure to

numerous minor chemicals that may be genotoxic or protective (Ames, *Science* 221:1256-1264 (1983); Ames, et al., *Proc. Natl. Acad. Sci. USA* 87:7777-7781 (1990); Ames, et al., *Proc. Natl. Acad. Sci. USA* 87:7782-7786 (1990); Carr, *Cancer* 55:218-224 (1985); Wattenberg, *Cancer Res. Suppl.* 43:2448s-2453s (1983); Wattenberg, L.W., *Cancer Res.* 45:1-8 (1985); Wattenberg, et al., *Diet, Nutrition and Cancer*:193-203 (1986); Tannenbaum, et al., *Adv. Cancer Res.* 1:451-501 (1953); National Research Council, *Diet, Nutrition and Cancer*, (1982); National Research Council, *Diet and Health: Implications for Reducing Chronic Disease Risk*, (1989); Creasey, *Diet and Cancer*, (1985); Knudsen, *Genetic Toxicology of the Diet*, (1986)). Rational recommendations for modifying human diets to reduce the risk of cancer require identification of dietary carcinogens and chemoprotectors, even though interactions among such factors in modulating cancer development are complex (Patterson, et al. *Am. J. Public Health* 78:282-286 (1988)). Whereas extensive efforts have been made to identify dietary carcinogens and mutagens (Ames, *Science* 221:1256-1264 (1983); Ames, et al. *Proc. Natl. Acad. Sci. USA* 87:7777-7781 (1990); Ames, et al., *Proc. Natl. Acad. Sci. USA* 87:7782-7786 (1990)), chemoprotective components have received far less attention.

Numerous epidemiological studies suggest that high consumption of yellow and green vegetables, especially those of the family Cruciferae (mustards) and the genus *Brassica* (cauliflower, cress, brussels sprouts, cabbage, broccoli), reduces the risk of developing cancer of various organs (Graham, et al., *J. Natl. Cancer*

Inst. 61:709-714 (1978); Graham, *Cancer Res. Suppl.* 43:2403s-2413s (1983);
Case 1:07-cv-07844-SAS Document 42-42 Filed 05/08/2008 Page 41 of 50
Colditz, et al., *Am. J. Clin. Nutr.* 41:32-36 (1985); Kune, et al., *Nutr. Cancer*
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Marchand, et al., *J. Natl. Cancer Inst.* 81:1158-1164 (1989); You, et al., *J. Natl.*
Cancer Inst. 81:162-164 (1989)). Moreover, administration of vegetables or of
some of their chemical components to rodents also protects against chemical
carcinogenesis (Wattenberg, *Cancer Res. Suppl.* 43:2448s-2453s (1983);
Wattenberg, *Cancer Res.* 45:1-8 (1985); Wattenberg, et al., *Diet, Nutrition and*
Cancer 193-203 (1986); Boyd, et al., *Food Chem. Toxicol.* 20:47-52 (1982)).

Well-documented evidence established that feeding of certain vegetables
(e.g., brussels sprouts and cabbage) induces both phase I and phase II enzymes²
in animal tissues (Conney, et al., *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 36:1647-
1652 (1977); Spornins, et al., *J. Natl. Cancer Inst.* 66:769-771 (1981); Spornins,
et al., *J. Natl. Cancer Inst.* 68:493-496 (1982); Aspary, et al., *Food Chem.*
Toxicol. 21:133-142 (1983); Bradfield, et al., *Food Chem. Toxicol.* 23:899-904

²Enzymes of xenobiotic metabolism belong to two families (i) phase I enzymes (cytochromes P-450), which functionalize compounds, usually by oxidation or reduction; although their primary role is to detoxify xenobiotics, several cytochromes P-450 can activate procarcinogens to highly reactive ultimate carcinogens (Miller, et al., *Bioactivation of Foreign Compounds*, 3-28 (1985)); and (ii) phase II enzymes, which conjugate functionalized products with endogenous ligands (e.g., glutathione, glucuronic acid, sulfate) and serve primarily a detoxification role (Jakoby, et al., *J. Biol. Chem.* 265:20715-20718 (1990)). Quinone reductase (QR) is considered a phase II enzyme because it has protective functions (Prochaska, et al., *Oxidative Stress: Oxidants and Antioxidants*, 195-211 (1991)) is induced coordinately with other phase II enzymes, and is regulated by enhancer elements similar to those that control glutathione transferase (Favreau, et al., *J. Biol. Chem.* 266:4556-4561 (1991)).

(1985); Salbe, et al., *Food Chem. Toxicol.* 24:851-856 (1985); Whitty, et al., *Food Chem. Toxicol.* 25:581-587 (1987); Ansher, et al., *Hepatology* 3:932-935 (1983); Ansher, et al., *Food Chem. Toxicol.* 24:405-415 (1986)) and stimulates the metabolism of drugs in humans (Conney, et al., *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 36:1647-1652 (1977); Pantuck, et al., *Clin. Pharmacol. Ther.* 25:88-95 (1979); Pantuck, et al., *Clin. Pharmacol. Ther.* 35:161-169 (1984)). The elevations of enzymes that metabolize xenobiotics may be highly relevant to the protective effects of vegetables, since relatively modest dietary changes not only affected the metabolism of drugs (Ansher, et al., *Food Chem. Toxicol.* 24:405-415 (1986)) but also modified the ability of carcinogens to cause tumors in rodents (Tannenbaum, et al., *Adv. Cancer Res.* 1:451-501 (1953); National Research Council, *Diet, Nutrition and Cancer* (1982); National Research Council, *Diet and Health: Implications for Reducing Chronic Disease Risk* (1989); Creasey, *Diet and Cancer* (1985); Knudsen, *Genetic Toxicology of the Diet* (1986); Longnecker, et al., *Cancer* 47:1562-1572 (1981); Fullerton, et al., *Proc. Am. Assoc. Cancer Res.* 29:147 (1988); Li, et al., *Cancer Res.* 50:3991-3996 (1990)). There is now very good evidence that when phase II enzymes are induced, animals and cells are protected against the toxic and neoplastic effects of carcinogens. In fact, anticarcinogens have been identified based on their ability to induce phase II enzymes. (Reviewed in Talalay (1992) "Chemical protection against cancer by induction of electrophile detoxication (phase II) enzymes" in *Cellular and*

There is a need in the art for the identification of specific compounds which are able to exert an anti-carcinogenic effect on mammals. Once identified, these chemoprotective compounds can be used as prophylactic medicaments or as food additives.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a pharmaceutical composition for cancer prevention.

It is another object of the invention to provide compounds which have cancer chemoprotection activity.

It is yet another object of the invention to provide a method for protecting against cancer development.

It is still another object of the invention to provide a food product which is supplemented with a chemoprotective compound.

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment a pharmaceutical composition for cancer prevention is provided. The composition comprises an active ingredient which is sulforaphane ((-)-1-isothiocyanato-(4R)-(methylsulfinyl)butane) (CAS 4478-93-7) or an analogue thereof, said analogue having a first moiety which is an isothiocyanate and a second moiety which is a polar functional group, wherein said analogue has a chain of one or more carbon

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atoms linking said first and said second moieties, and wherein said analogue contains no pyridyl moieties.

In another embodiment of the invention compounds are provided which have cancer chemoprotection activity. The compounds include: 1-isothiocyanato-5-methylsulfonylpentane ($\text{CH}_3\text{-SO}_2\text{-(CH}_2)_5\text{-NCS}$) ((GHP 1003), 6-isothiocyanato-2-hexanone ($\text{CH}_3\text{CO(CH}_2)_4\text{NCS}$) (GHP 1105), *exo*-2-acetyl-6-isothiocyanatonorbornane (GHP 1066), *exo*-2-isothiocyanato-6-methylsulfonylnorbornane (GHP 1068), 6-isothiocyanato-2-hexanol (GHP 1106), 1-isothiocyanato-4-dimethylphosphonyl-butane (GHP 1078), *exo*-2-(1'-hydroxyethyl)-5-isothiocyanatonorbornane (GHP 1075), *exo*-2-acetyl-5-isothiocyanatonorbornane (GHP 1067), and *cis*- or *trans*-3-(methylsulfonyl)cyclohexylmethylisothiocyanate (GHP 1080 and 1079).

In yet another embodiment of the invention a method for protecting against cancer induction or progression is provided. The method comprises the step of: administering to a mammal a chemoprotective composition consisting essentially of sulforaphane ((-)-1-isothiocyanato-(4R)-(methylsulfinyl)butane) or an analogue thereof, said analogue having a first moiety which is an isothiocyanate functionality and a second moiety which is a polar functional group, wherein said analogue has a chain of one or more carbon atoms linking said first and said second moieties, and wherein said analogue contains no pyridyl moieties, in an amount effective in producing a cancer preventive effect.

In still another embodiment of the invention a food product is provided. The product has been supplemented with an active chemoprotective compound, wherein said compound is sulforaphane ((-)-1-isothiocyanato-(4R)-(methylsulfinyl)butane) or an analogue thereof, said analogue having a first moiety which is an isothiocyanate functionality and a second moiety which is a polar functional group, wherein said analogue has a chain of one or more carbon atoms linking said first and said second moieties.

These and other objects of the invention provide the public with positive means to lower the risk of developing cancers.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of sulforaphane.

Figure 2 shows the fragmentation pattern of sulforaphane.

Figure 3 shows the synthesis of (GHP 1003).

Figure 4 shows the synthesis of (GHP 1066 and 1067).

Figure 5 shows the synthesis of (GHP 1068).

Figure 6 shows the synthesis of (GHP 1073).

Figure 7 shows the synthesis of (GHP 1075).

Figure 8 shows the synthesis of (GHP 1078).

Figure 9 shows the synthesis of (GHP 1079 and 1080).

Figure 10 shows the synthesis of (GHP 1105).

Figure 11 shows the synthesis of (GHP 1106).

DETAILED DESCRIPTION OF THE INVENTION

Chemoprotective activities have been detected in certain vegetables which are able to induce the activity of enzymes that detoxify carcinogens (phase II enzymes). One such activity has been detected in broccoli which induces quinone reductase activity and glutathione S-transferase activities in murine hepatoma cells and in the organs of mice. This activity has been purified from broccoli and identified as sulforaphane. Analogues of sulforaphane have been synthesized to determine structure-function relationships.

It is the discovery of the present invention that sulforaphane and its isothiocyanate analogues have chemoprotective activity in excess of previously discovered compounds. The analogues contain a moiety which is a polar functional group. This may be, for example, a sulfoxide, a ketone, a sulfone, a sulfide, a thioester, a thioether, a nitrile, a nitro, a carboxylic ester, a carboxylic acid, a halogen, a phosphine oxide, or a hydroxyl group. The isothiocyanate moiety and the polar functional group are linked by a chain of one or more carbon atoms. Preferably there are at least three carbon atoms in the chain. Typically there are three to five carbon atoms in the chain. The analogues do not contain pyridyl moieties.

The chemoprotective compounds of the present invention can be administered to mammals as a prophylactic against chemically induced cancers. The compounds can be formulated in suitable excipients for oral administration, for topical administration, or for parenteral administration. Such excipients are

mammals. Typically they are sterile, and contain no toxic, carcinogenic, or mutagenic compounds which would cause an adverse reaction when administered. Administration of the compounds can be performed before, during, or after exposure to the offending carcinogens or procarcinogens. Suitable doses to be administered are those which are sufficient to induce a demonstrable increase of phase II enzymes. This will typically not exceed 500 μ moles per kg per day, but may be much lower.

Sulforaphane and sulforaphene are known to be produced by plants, such as hoary cress, radish and other plants (Mislou, et al. (1965) *J. Am. Chem. Soc.* 87:665-666; Schmid, et al. (1948) *Helvet. Chim. Acta* 31:1017-1028; Hansen et al. (1974) *Acta Chem. Scand. Ser. B* 28:418-424). For the purposes of the present invention, they can be isolated from plants or synthesized. Bertoin, alyssin, erucin, erysolin, iberiverin, iberin, and cheirolin can also be isolated from plants; these compounds appear to be less active as inducers than sulforaphane and sulforaphene, at least in cell culture.

Other synthetic analogues of sulforaphane will preferably not be heteroaromatic and more preferably will not be aromatic. Such analogues include olefins, aliphatics, and non-aromatic ring compounds. Some examples of these are shown below in Table 3. The CD value provides a measure of the potency of the compounds as inducers of phase II enzymes, specifically quinone reductase. The

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preferred compounds of the invention have CD values less than 1, although some established chemoprotectors have higher CD values.

Other analogues of sulforaphane can be used which are not specifically shown. The relative ability of the compound to induce the chemoprotective enzymes can be assessed as taught below, either by testing induction in cell lines, or in whole animals. The compounds can also be tested for the ability to suppress hepatoma formation in rats by 3-methylcholanthrene, 2-acetylaminofluorene, diethylnitrosamine, *m*-toluenediamine, and azo dyes. They can also be tested for the ability to block the neoplastic effects of diethylnitrosamine or benzo[*a*]pyrene on lung and forestomach of mice or of dimethylbenz[*a*]anthracene (DMBA) on mammary tumor formation in rats.

Also provided by the present invention are food products which have been supplemented with a chemoprotective compound of the present invention. The supplement may be isolated from plants or synthesized.

EXAMPLES

Example 1

This example describes the rapid cell culture assay which was used in the purification of a chemoprotective compound from broccoli.

Assay of Inducer Potency. Inducer activity was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates (Prochaska and Santamaria, *Anal. Biochem.* 169:328-336 (1988); Prochaska et al., *Proc. Natl. Acad. Sciences USA* 89:2394-2398 (1992)). QR activity (based on the formati n

of the blue-brown reduced tetrazolium dye) was measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and the cell density was determined in the second plate by staining with crystal violet. Quantitative information on specific activity of QR, the inducer potency, and the cytotoxicity of the extract or compound tested is obtained by computer analysis of the absorbances. One unit of inducer activity is defined as the amount that when added to a single microtiter well doubled the QR specific activity. The CD value is the concentration of a compound required to double the quinone reductase specific activity in Hepa 1c1c7 murine hepatoma cells.

Sources of Vegetable and Preparation of Extracts. Vegetables were homogenized with 2 vol of cold water in a Waring Blendor at 4°C. The resultant soups were lyophilized to give dry powders, which were stored at -20°C. Portions (400 mg) of these powders were extracted for 6-24 hr with acetonitrile by shaking in glass vessels at 4°C. The extracts were filtered and evaporated to dryness. The residues were dissolved or suspended in acetonitrile or dimethyl formamide.

The specific activities of QR were raised nearly 6-fold at the highest extract concentrations tested, at which less than 20% cytotoxicity was observed. The inductions obtained with broccoli and with other vegetable extracts were proportional to the quantity of extract added over a reasonably wide range. The toxicities of these extracts were modest and were unrelated to their inducer potencies.

Extracts of a series of organically grown vegetables cultivated under a variety of conditions showed large differences in inducer potencies. Although many vegetable extracts induced QR, certain families were consistently more potent inducers. For example, where extracts of several Cruciferae had potent inducer activity, extracts of Solanaceae (peppers, potatoes, tomatoes) had low inducer activity. Of the 24 vegetables examined only 6 showed detectable toxicity; the others were nontoxic at the highest concentrations tested.

Cytotoxicity measurements are important because phase II enzyme inducers may be toxic and/or carcinogenic. Moreover, by use of mutant Hepa cells defective in aryl hydrocarbon receptor or cytochrome P-450 function (Zhang, et al., *Proc. Natl. Acad. Sci. USA* 89:2399-2403 (1992); Prochaska, et al., *Cancer Res.* 48:4776-4782 (1988); De Long, et al., *Carcinogenesis* 8:1549-1553 (1987)), our assay system can distinguish *monofunctional* inducers (which elevate phase II enzymes selectively), from *bifunctional* inducers (which elevate both phase I and II enzymes) (Prochaska, et al., *Cancer Res.* 48:4776-4782 (1988)). Such information is crucial for identification of chemoprotective enzyme inducers for potential use in humans. Ideally, such inducers should be monofunctional, because elevated activities of phase I enzymes may lead to carcinogen activation. The assay of phase II enzymes makes possible further detailed analysis of the effects of treatment of vegetables (e.g., breeding, mutagenesis, growth, storage, and cooking conditions) that might enhance or depress such induction.